In Vitro Scaffold Construction for a Bio-Artificial Liver

BEE 453
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Jeff Wang, Sharon Chow
May 7, 2004

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Executive Summary:

The main focus of this investigation is to design a scaffold that will accommodate a growing Bio-Artificial Liver (BAL) with oxygen. The two design objectives are to find the maximum length and the distance between the artificial capillaries of the scaffold to provide adequate oxygen supply above 1.98×10^{-19} g/um^3 to prevent hypoxia to the growing liver tissues. By utilizing industrial modeling software, FIDAP and GAMBIT, a model of a single capillary with liver tissue attached directly was constructed to simulate the oxygen delivery by means of diffusion and convection from the capillary wall to the tissue and the uptake by metabolism. From the results obtained, it was concluded that diffusion, not convection of the oxygen flow within the capillary was the dominant process of oxygen transport throughout the tissue. The maximum distance traveled into the tissue with capillary length of 60 μm was 147 μm from the capillary at the inlet side of the tissue while diffusion at the outlet tissue was at a modest 108 μm. These values are unacceptable for the feasible construction of oxygen transport system solely based on diffusion. Thus, this investigation concludes that novel methods of greater complexity are needed to construct a more efficient and economically applicable oxygen delivery system for the mass production of bio-organs.

Introduction and Design Objectives:

As of April 2004, there are a total of 90,438 patients on the waitlist for organ transplantation with waiting time that can take up to six years. Further adding to the difficulty of receiving an organ from a donor, the organ transplantation availability depends on age, diagnosis, blood type, ethnicity, and other compatibility issues of both the patients and the donor. Hence, the development of an artificial bio-organ is critical in the 21st Century.

A need to replace liver transplantation as a solution for acute liver failure exists since the unavailability of donor organs results in 30,000 deaths per year. The liver is among the 2nd highest demand (17 000), following the kidney. Since liver cells have a high regenerative capacity and the graft survival rate for liver transplants are among the lowest, we find this high demand coupled with construction possibility for a Bio-Artificial Liver (BAL). Adhering to these principles, the design objective for this project will be to develop a system for the growth of a bio-artificial liver (BAL) in vitro. A vertical scaffold to simulate a network of capillaries will deliver oxygen among other nutrients to maturing liver cells. The proposed model is illustrated below in Fig.1.

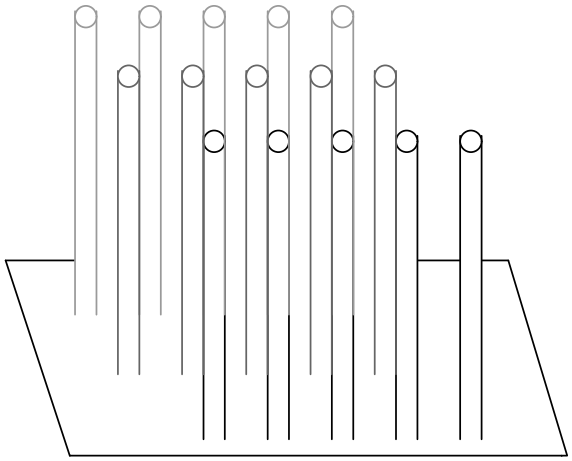

Figure1: Proposed scaffold capillary network for BAL design

As nano-biotechnology further develops, diffusion-limited mass flow will have to be overcome to create more efficient nano-fabricated devices. These limits are seen in the field of tissue engineering in that oxygen delivery is limited to diffusion. Naturally, oxygen delivery in the body still relies on diffusion. However capillaries deliver oxygen to the tissue through the blood. As the blood goes through the capillary, oxygen diffuses into the surrounding tissue. Therefore since it still relies on diffusion capillaries cannot be spaced very far apart, otherwise hypoxia would ensue. Hypoxia is the deficiency of oxygen delivery, and a conservative value of 60mmHg of oxygen was taken as the point where hypoxia begins.

This project focuses on the oxygen delivery from the capillaries to the tissue of the proposed model (Fig.1). Oxygen is essential to sustain metabolism in cells, and therefore, crucial to the design parameters of the scaffold. The tissue cells are modeled to surround the capillaries

directly, and the distance between the capillaries as well as the length of the capillaries is our main objectives for this investigation. By modeling the scaffold in GAMBIT and FIDAP, we can see how oxygen concentration varies along, and away from the capillary. We construct an axi-symmetric model in cylindrical co-ordinates and add in factors including velocity of the oxygen-carrying blood source at the capillary inlet, the diffusion rate in tissues as well as the metabolic reaction for oxygen consumption in the tissue. By taking the point where the level of hypoxia begins, and its distance away from the capillary inlet, we can then deduce the minimum distance between capillaries and the length of each network of capillaries.

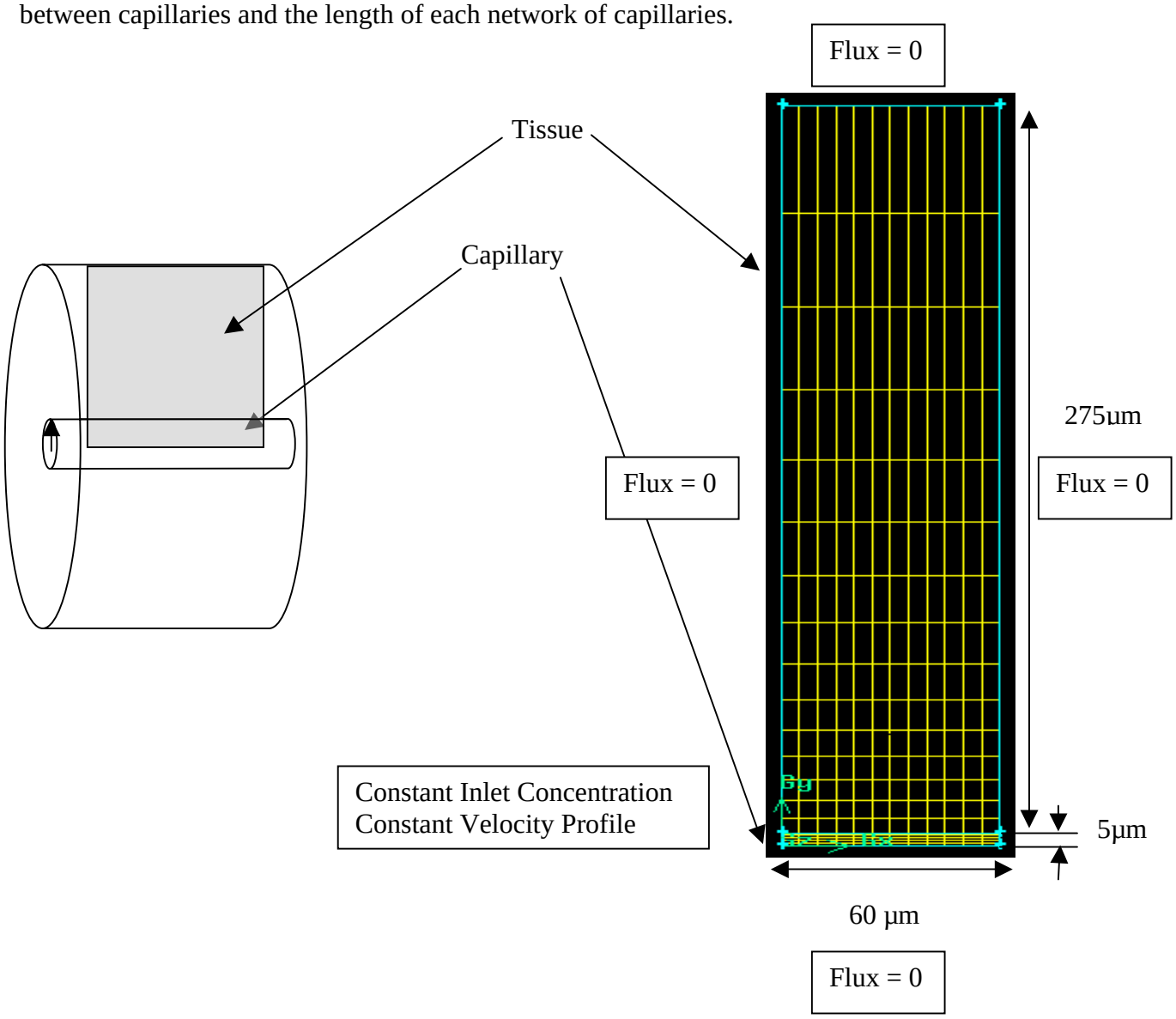

Figure 2: Model Schematics with proposed mesh and boundary conditions.

Results and Discussion:

Quantitative Results

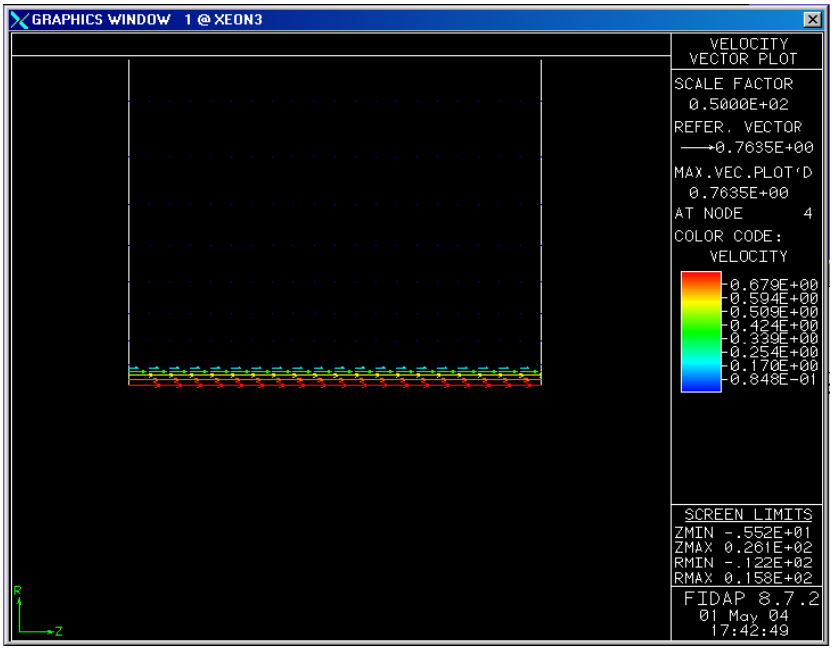

Figure 3: Velocity Profile in the Capillary remains constant

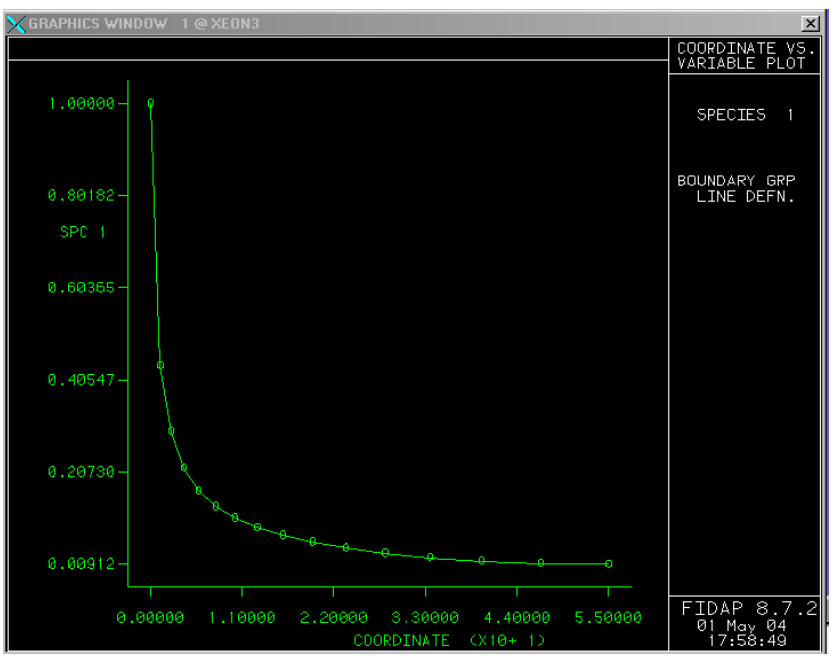

Figure 4: Left Tissue concentration profile where hypoxia occurring at 147 µm

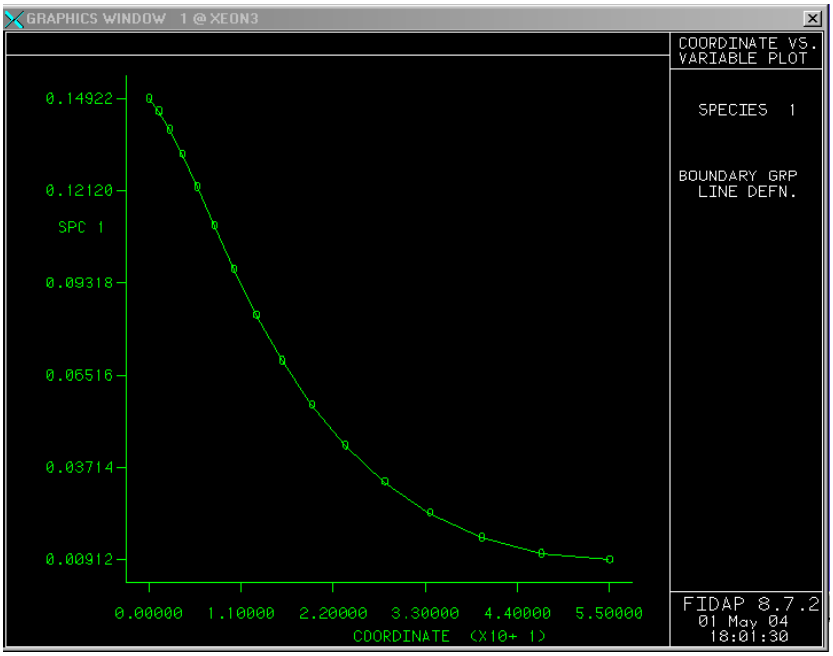

Figure 5: Right Tissue concentration profile where hypoxia occurring at 108 µm

The velocity profile throughout the length of the capillary is laminar, parabolic flow. This velocity profile can be seen in Fig 3, where the strongest flow is present at the bottom of the mesh, which represents the center of the capillary in an axi-symmetric model.

In order to access the depth of O_2 diffusion within the tissue, the left tissue, which represents the maximum amount of diffusion depth and the right tissue, which represents the minimum diffusion, is chosen. Fig. 4, representing the left tissue, shows that hypoxia, which occurs at 1.98 x 10^{-19} g/um^3 of O_2, occurs at 147µm away from the capillary. Similarly, Fig.5 shows that hypoxia occurs at 108 µm away from the capillary at the right tissue. By accessing the extremes, the minimum possible length of tissue between each adjacent capillaries of the scaffold was determined to be 108 µm. In both cases, it is important to note that the capillary length was 60 µm.

Mesh convergence

Original Results

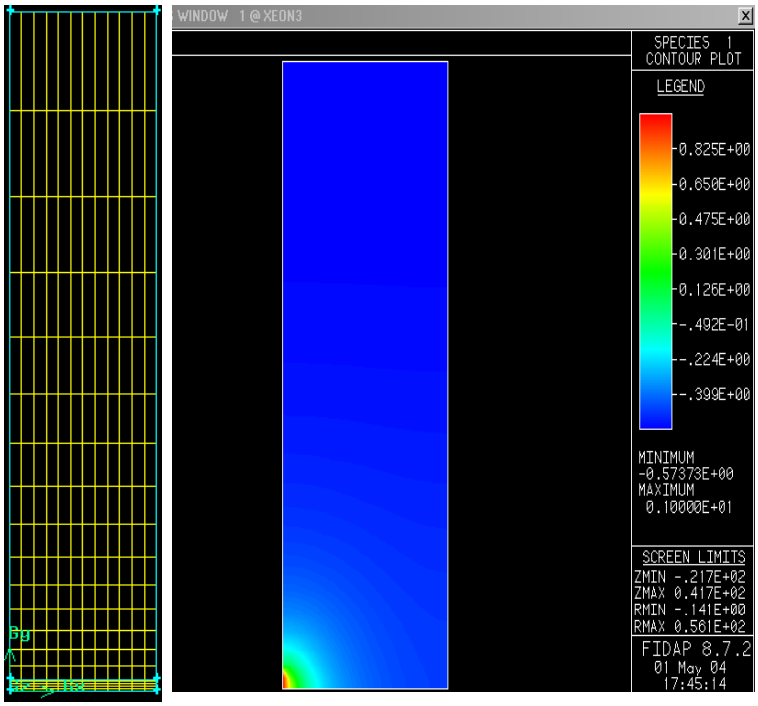

Figure 6: Results from original mesh

Refined Mesh

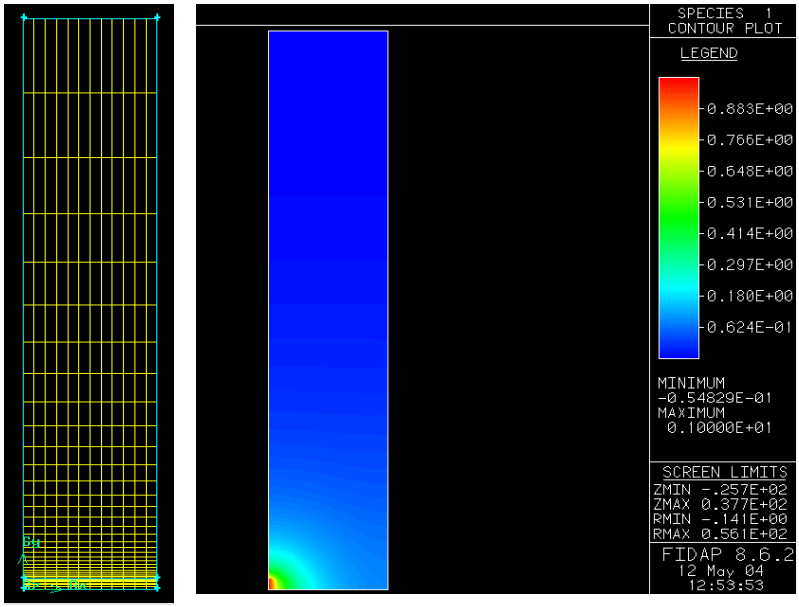

Figure 7: Results from refined mesh.

The hypoxia level from the original mesh for the left side of the tissue was 147µm. Fig. 6 represents the species contour and the mesh for the unrefined mesh. Fig. 7 represents the refined mesh. Here the hypoxia level at the left side of the tissue was 140 µm. The difference can be attributed to more nodes.

Qualitative Explanation of Results

Our model consists of blood, pre-saturated with oxygen from the lungs, flowing into a capillary surrounded by tissue of a constant density. The modeled flow is parabolic in shape with the maximum velocity occurring at the center of the capillary and a velocity of zero at the capillary wall. This goes against the common tube model known as the Haynes profile where the flow is tubular in form instead of parabolic. Previous studies have shown that blood flow in capillaries tends to follow this tubular or trapezoidal form rather than parabolic. This parabolic flow is assumed constant throughout the length of the capillary. The flow model also ignores the possibility of red blood cells (RBC) stacking on top of each other in stacks known as Rouleux formations, and it ignores 'tank treading' that can occur if the RBC begin rotating as it flows. Therefore this leads to the major assumption of constant blood density. Blood is indeed non-Newtonian, but our model assumes that it behaves as a Newtonian fluid; which gives us the constant viscosity (at a hematocrit of 45).

Our problem definition only concerns itself with the steady state solution. The reasoning for this is that the tissue and capillary are always filled with blood and oxygen of a given concentration. The only time the non-steady state solution result would occur would be during development of the tissue and capillary themselves. Therefore only the steady state solution is of interest. Of course this solution would be subject to change for different flow rates such as would occur during strenuous exercise.

The tissue in our model is allowing oxygen to diffuse into it from the capillary. As the oxygen diffuses into the tissue, it is metabolically used up as the tissue performs metabolism to create ATP. The model assumes a uniform tissue density composed of the same cells. Therefore our reaction rate is constant. Our reaction model also assumes the reaction is zero order. Our model also assumes a constant diffusion of oxygen into the tissue, but in reality the behavior of hemoglobin would make this assumption unrealistic. Hemoglobin loses oxygen at a nonlinear rate due to allosteric changes in the shape of the protein.

Our model shows that diffusion is the dominant term over advection. Therefore the model has oxygen diffusing out of the capillary only near the inlet instead of along the entire capillary. The model shows that hypoxia is occurring towards the top of the mesh at about 147 um into the tissue. Former research performed by Krogh gave a value of 250 um. So our model is fairly consistent to this famous result. Our sensitivity analysis will show that this value of 147 um is highly dependent on inlet concentration, capillary length, and our reaction rate.

Sensitivity Analysis

To evaluate which parameters could influence oxygen transport significantly, we performed sensitivity analysis on capillary length, reaction rate, velocity, and diffusivity. Recall as under normal conditions, the distance from the capillary at which the concentration drops to 60 mmHg is 147 µm. Each parameter was altered +/- 15% and the distance from the capillary where hypoxia occurs (P = 60 mmHg, C = 1.98 E -19) at the left tissue (*z* = 0) was estimated. Results are listed and plotted below. The results of sensitivity analysis are summarized in Table 1 and Fig. 8 for the four variables.

Table 1: Sensitivity Analysis

Parameter	% Deviation	Distance (µm)
Length	+15.00%	15
Reaction Rate	-15.00%	R > 275
	+15.00%	44.2
Velocity	-15.00%	R > 275
	+15.00%	105
Diffusivity	-15.00%	118
	+15.00%	R > 275

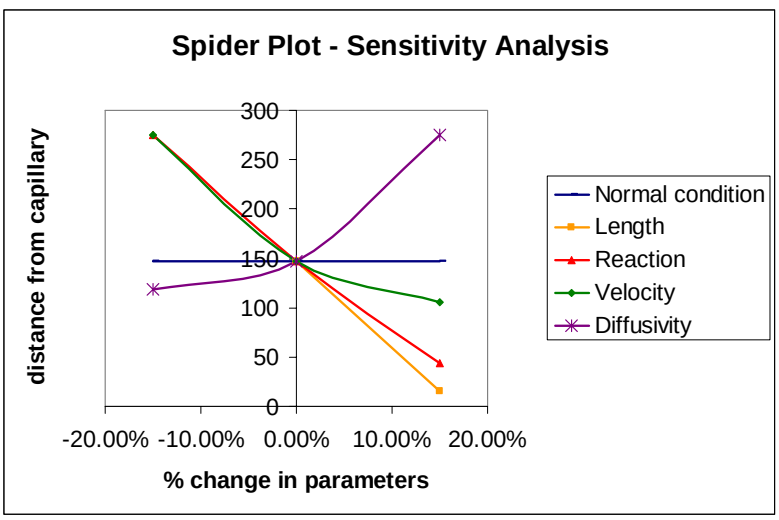

Figure 8: Spider Plot of Sensitivity Analysis

For some of the parameters, the height where hypoxia occurs could not be determined because it was greater than 275 micrometers. It is noted that there is some uncertainty as to why there are asymmetrical influences about the mean and suggest that for further analysis to take incremental deviations less than 15%. From this analysis it is probable that the length of the capillary has a significant influence on the distance at which hypoxia occurs. This is understandable as the capillary lengthens; it allows more oxygen to diffuse in the axial direction. The next most sensitive parameter is the reaction rate; which confirms that oxygen concentrations depend on how fast cells are consuming resources. Diffusivity is not expected to change significantly and the velocity is not a dominant process in transport and thus does not alter results on a large scale.

Discussion on Manufacturability, Sustainability, and Social Implications:
If our design concept is to be made reality, several constraints that concern health and safety, manufacturing, and sustainability must be solved.

The small dimensions of our design represent a manufacturing and sustainability challenge. Advancements in micro-fabrication techniques will aid in constructing channels to the required scale. However, the walls of our scaffold must like a semi-permeable membrane with pores. Oxygen must be allowed to diffuse out into the tissue, while restricting blood or plasma to inside the capillary. Carbon dioxide, a waste product of cellular metabolism, must be removed from the tissue in order to guarantee cell sustainability. This kind of molecular separation has been demonstrated in environmental cleanup procedures but integrating this technology with micro-fabrication may be complex.

One of the most hotly debated aspects regarding the development of BAL devices surrounds the choice of cell types. Choices have been narrowed down to human hepatocytes or porcine hepatocytes, each with their own intrinsic benefits and disadvantages. From a sustainability standpoint, human hepatocytes grow poorly in cell media, making it difficult to accumulate enough cells to produce an effective BAL. However, the bio-chemicals they produce are the same ones manufactured by a natural liver so the chance of immunity rejection is less. Porcine hepatocytes are easier to grow and actually have shown a better ammonia clearing ability than human cells. On the other hand, porcine cells do pose an increased health risks, as they are susceptible to porcine endogenous retroviruses.

This project attempts the noble goal of solving the major problem health officials and patients face related to organ transplantation. There are currently thousands of patients waiting for organs that must match their specific condition. Not only must blood type match, but those that eventually receive the organ are unfortunately chosen based on age, wealth, and in many cases ethnicity. The current system favors giving organs to those that can afford adequate health care, and elderly patients are usually overlooked in favor of younger patients. Current statistics show that African-American male patients are the least likely to receive potential organs. If this study's ultimate goal were achieved artificial organs could help alleviate the major drain of resources that organ transplants now consume.

Conclusion:
Using FIDAP, we found the distance between capillaries for a scaffold designed to deliver oxygen to a Bio-Artificial liver. The momentum and species equations were solved to find a steady state solution. Based on the parameters utilized in the study, the channels must be at a range of 108-147 μm apart for optimum tissue growth when the capillary distance is 60 μm. Diffusion was found to be the primary factor in oxygen transport, rather than advection. Thus, our original concept design consisting of long slender tubes needed to be changed. Stacked layers consisting of short, thick capillaries (left) represent the ideal configuration for our model.

Future work remains on this project. First, sensitivity analysis indicates that our results are highly dependent on the values of the constants used in the governing equations. Further refinement is needed. Secondly, several constraints affecting the health and safety, sustainability, and the scaffold's manufacturing process must be considered.

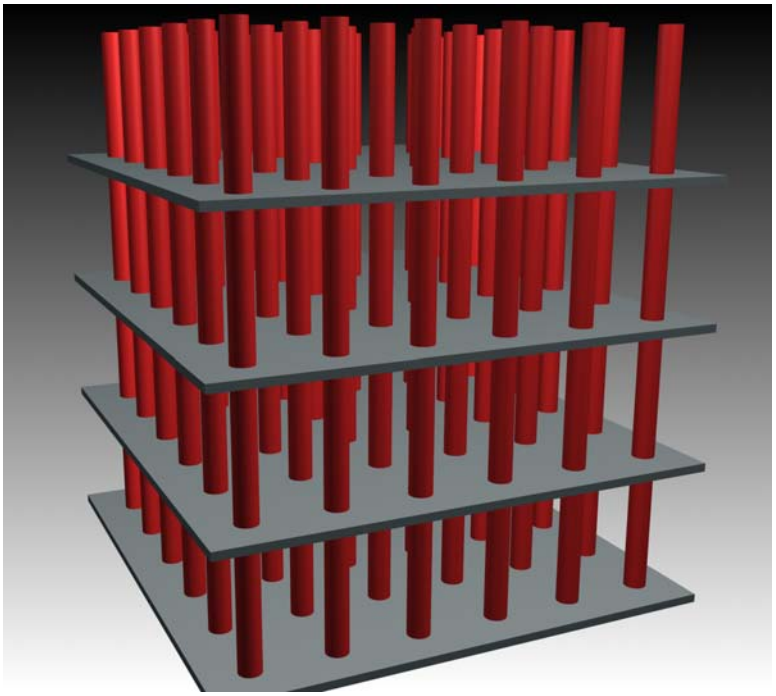

Figure 9: Proposed Scaffold Model

Appendix A: Model Derivation and Assumptions

A model of a single capillary attached to tissue was created to generate a simulation of the oxygen transport across the tissue bound by adjacent capillaries. The model was simplified and only one capillary and its surrounding tissue was taken into consideration.

The schematic of simplified two-capillary problem is as follows:

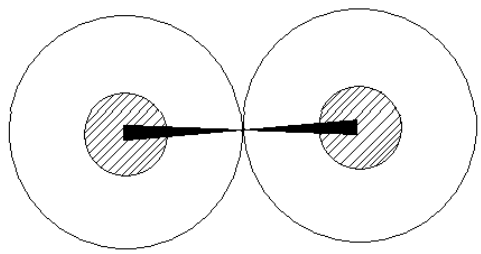

Figure 10: Hypothesized oxygen gradient between two adjacent capillaries with highest O_2 concentration at the center of the capillaries

Simplifying to a single capillary:

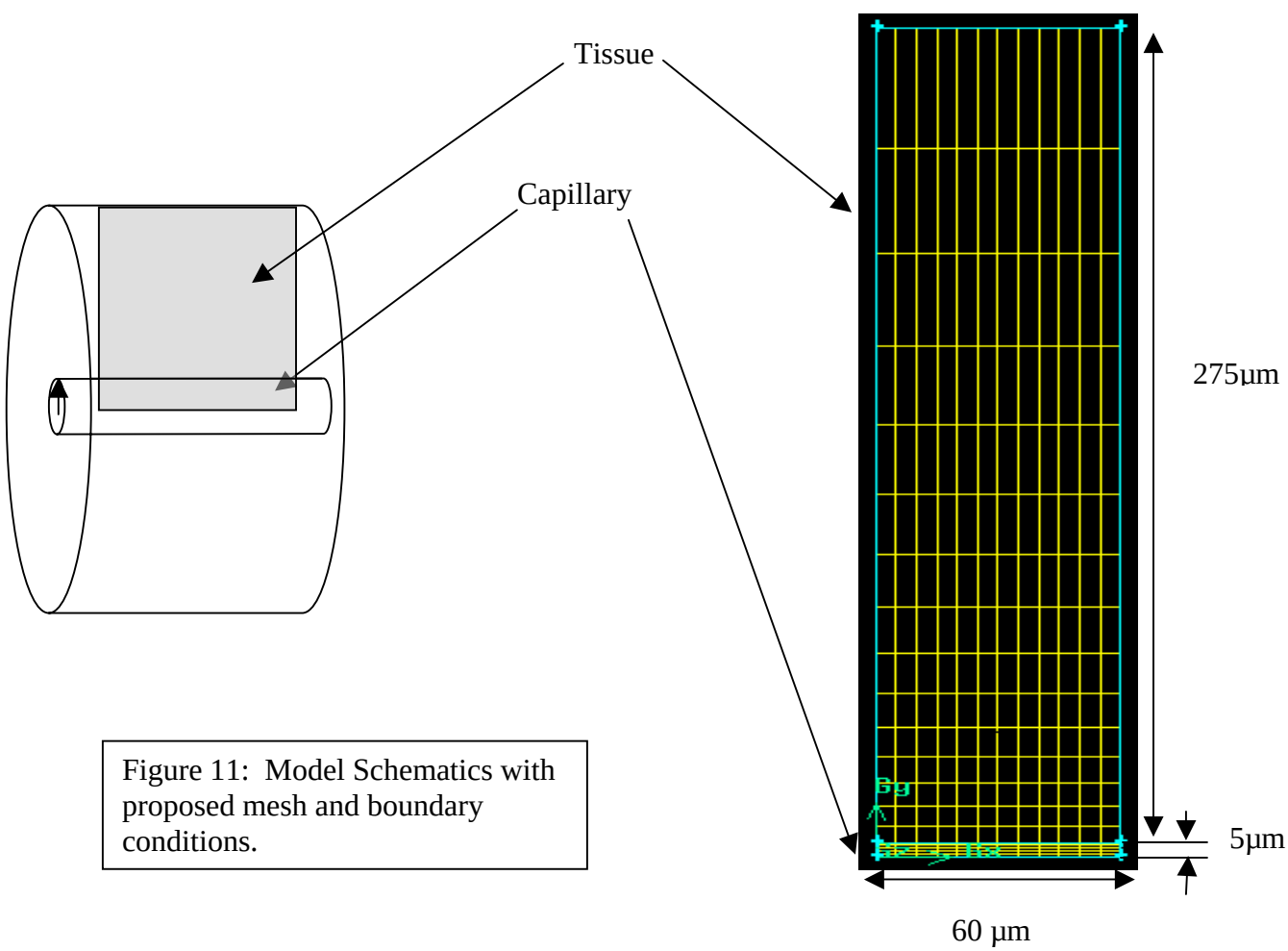

Figure 11: Model Schematics with proposed mesh and boundary conditions.

Governing Equations

For transport of oxygen:

$$\frac{\partial C}{\partial t} + u_z \frac{\partial C}{\partial z} = D_{rz}\left(\frac{\partial^2 C}{\partial r^2} + \frac{\partial^2 C}{\partial z^2}\right) + R$$

For blood flow:

$$\rho\left(\frac{\partial u_z}{\partial t} + v_z \frac{\partial u_z}{\partial z}\right) = \mu\left(\frac{\partial^2 u_z}{\partial z^2}\right) - \frac{\partial p}{\partial z}$$

$$u_z = u_{\max}\left(1 - \frac{r^2}{R^2}\right)$$

Boundary Conditions

For $z = 0$ and $z = 60$
(Left tissue and Right tissue)

$$\left.\frac{\partial C}{\partial z}\right|_{r=5}^{275} = 0$$

$$u_z = u_{\max}\left(1 - \frac{r^2}{R^2}\right) \text{ for } 0 < r < 5$$

otherwise $u = 0$

For $r = 275$
(Far Tissue)

$$\left.\frac{\partial C}{\partial r}\right|_{z=0}^{60} = 0$$

For $r = 0$
(Axis)

$$\left.\frac{\partial C}{\partial r}\right|_{z=0}^{60} = 0$$

Boundary at tissue and capillary: V = 0

$$\frac{\partial u}{\partial r} = 0 \quad \text{in the capillary}$$

$C_i = 1$ concentration at the inlet of capillary

Reaction Term:

Zeroth – order reaction term that is a constant.

Summary of Assumptions:

1. All capillaries have uniform geometry of a perfect cylinder of 5 micrometers.
2. All simulation proceeds within our defined constants of diffusivity and density.
3. The tissue is directly adjacent to the capillary with no other gaps in between.
4. The oxygen carrying fluid and tissue are assumed to have constant properties in terms of viscosity, density, flow-rate and inlet oxygen concentration.
5. There is no flow in r-direction but only in z-direction of fluid inside the capillary.
6. Zeroth – order reaction for O_2 metabolism by liver tissue.
7. Steady State Equation.
8. Constant O_2 concentration at the inlet.
9. Rigid boundary layers.
10. Axi-symmetric model.
11. Laminar fluid flow inside the capillary.

Appendix B: Mathematical Statements

Table 2: Spreadsheet of all dimensional constants and their respective non-dimensional equivalents

	Value (dimensional)	Value* (non-dimensional)
Blood Density[1]	1.06×10^{-12} g/um^3	1
Blood Viscosity[-2]	5.94×10^{-5} g/ um * sec	320215
$\text{Diffusivity}_{\text{Tissue}}$[4]	1750 um^2/sec	1
$\text{Diffusivity}_{\text{Capillary}}$[5]	1060 um^2/sec	.606
Inlet Concentration[4]	4.5×10^{-18} g/um^3 (100 mmHg)	1
Maximum Velocity[3]	260 um/sec	.7429
Reaction rate constant[5]	-6.10×10^{-21} g/um^3 * sec	-3.2×10^{-5}
Level till hypoxia[4]	1.98×10^{-19} g/um^3 (60 mmHg)	0.044

Methods for making constants nondimensional*:

$$\text{Blood density*} = \frac{\text{Blood density}}{\text{Blood density}} = 1$$

Characteristic length = 5 um

$$\text{Need variable } u_{00} = \frac{D_{cap}}{L} = 1750/5 = 350 \text{ um/sec}$$

$$\text{Blood viscosity*} = \frac{\text{Blood viscosity}}{(\text{Blood dens.})(u_{oo})(L)} = 320215$$

$$\text{Diffusivity}_{\text{Tissue}}\text{*} = \frac{\text{Diffusivity}_{\text{Tissue}}}{\text{Diffusivity}_{\text{Capillary}}} = 0.606$$

$$\text{Diffusivity}_{\text{Capillary}}\text{*} = \frac{\text{Diffusivity}_{\text{Capillary}}}{\text{Diffusivity}_{\text{Capillary}}} = 1$$

$$\text{Inlet Concentration*} = \frac{\text{Inlet Concentration}}{\text{Inlet Concentration}} = 1$$

$$\text{Maximum Velocity*} = \frac{\text{Maximum Velocity}}{u_{00}} = .7429$$

$$\text{Reaction rate constant**} = \frac{(\text{Reaction rate constant})(L^2)}{\text{Diffusivity}_{\text{Tissue}}} = 1.44 \times 10^{-22} \text{ g/um}^3$$

$$\text{Reaction rate constant*} = \frac{\text{Reaction rate constant**}}{\text{Inlet Concentration}} = -3.2 \times 10^{-5}$$

$$\text{Level till hypoxia*} = \frac{\text{Level till hypoxia}}{\text{Inlet Concentration}} = 0.044$$

(at this non-dimensional concentration on the concentration plots is where hypoxia occurs)

Appendix C: FIDAP Commands

PROBLEM Command
Axis-Symmetric (Geometry Type): The capillary and tissue system was modeled as slab axis-symmetric.
INCOMPRESSIBLE FLUID (FLOW REGIME): Fluid flow inside the capillary was modeled to be incompressible.
STEADY STATE (SIMULATION TYPE): The problem can be assumed to be steady state due to the constant reaction term and inlet concentration that doesn't change with time.
LAMINAR (FLOW TYPE): Fluid flow inside the capillary is considered to be a laminar flow.
NON-LINEAR (CONVECTIVE TERM): Convection was assumed to be non-linear inside the capillary
MOMENTUM (MOMENTUM EQN): Fluid inside the capillary was modeled to be Newtonian.
ISOTHERMAL (ENERGY EQN): The solution was not dependent upon temperature.
FIXED (SURFACE TYPE): The edges of the mesh were assumed to be stationary.
NOSTRUCTURAL (STRUCTURAL SHAPE): The mesh is unstructured.
NOREMESHING (ELASTICITY REMESHING): The mesh did not change shape during the simulation.
SPECIES: Oxygen Diffusion was the only species used in the mass equation.
SINGLE PHASE (NUMBER OF PHASES): There were no phase changes.

SOLUTION Command*
SEGREGATION: Set to 100
VELOCITY CONVERGENCE: Set at 1.0E-2
RESC: Set at 1.0E-1
SOLUTION CHANGE: Set at 0
RELAXATION: Set at 3, 3, 0, 5, 5, 0, 0, 0, 3, 3
*Data provided by FIDAP consultant for convergence of velocity profile within the capillary

```
PROB (AXI-, INCO, STEA, LAMI, NONL, NEWT, MOME, ISOT, FIXE, NOST, NORE,
    SING, SPEC = 1.0
PRES (MIXE = 0.100000000000E-15, DISC)
EXEC (NEWJ)
SOLU (SEGR = 100, VELC = 0.100000000000E-03, RESC = -0.100000000000E-02,
     SCHA = 0.000000000000E+00)
RELA (HYBR)
  0.3000000000E+00, 0.3000000000E+00, 0.0000000000E+00, 0.5000000000E+00,
  0.5000000000E+00, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
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  0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
ENTI (NAME = "Capillary", FLUI, MDEN = "capdens", MVIS = "capvisc", SPEC = 1.0,
     MDIF = "capdiff")
ENTI (NAME = "Tissue", SOLI, SPEC = 1.0, MDIF = "tissdiff", MREA = "metabolism")
ENTI (NAME = "Axis", PLOT)
ENTI (NAME = "Outlet", PLOT)
ENTI (NAME = "Boundary", PLOT)
ENTI (NAME = "Inlet", PLOT)
ENTI (NAME = "Left Tiss", PLOT)
ENTI (NAME = "Far Tissue", PLOT)
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```
ENTI (NAME = "Right Tissue", PLOT)
DENS (SET = "capdens", CONS = 1.0)
VISC (SET = "capvisc", CONS = 320215.0)
DIFF (SET = "capdiff", CONS = 1.0)
DIFF (SET = "tissdiff", CONS = 0.606)
REAC (SET = "metabolism", CONS, TERM = 1, KINE)
  -0.3200000000E-04, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
   0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
   0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
   0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00,
   0.0000000000E+00, 0.0000000000E+00, 0.0000000000E+00
BCNO (URC, ENTI = "Inlet", CONS = 0.000000000000E+00)
BCNO (SPEC = 1.0, ENTI = "Inlet", CONS = 1.0)
BCNO (VELO, ENTI = "Boundary", CONS = 0.000000000000E+00)
BCNO (UZC, ENTI = "Inlet", POLY = 1, SYST = 1, CYLI)
   0.7429000000E+00, -0.7429000000E+00, 0.2000000000E+01, 0.0000000000E+00,
   0.0000000000E+00
BCFL (SPEC = 1.0, ENTI = "Left Tiss", CONS = 0.000000000000E+00)
BCFL (SPEC = 1.0, ENTI = "Far Tissue", CONS = 0.000000000000E+00)
BCFL (SPEC = 1.0, ENTI = "Right Tissue", CONS = 0.000000000000E+00)
```

Appendix E: References

World Health Organization (WHO)
World Heath Report 2003: Deaths by cause, sex and mortality stratum in WHO regions
Source: http://www.who.int/whr/2003/en/Annex2-en.pdf

Asian Liver Center at Stanford University
Source: http://liver.stanford.edu/index2.asp?lang=eng&page=statistics

DSP Development Corporation
Source: www.dadisp.com/spring98/ spr98c.htm

Appendix 1 constants:
[1]N. Tsuda, K, Kuroda and Y.Suzuki. An Inverse Method to Optimize Heating Conditions in RF capacitive hyperthermia, IEEE, transactions in Biomedical Engineering, 1996m 43 (10), 1032

[2]Chien S. "Shear dependence of Effective Cell Volume as a Determinant of Blood Viscocity." Science. 168, 22 May 1970

[3]http://www.sp.uconn.edu/~bi107vc/sp02am/toedt/lecture6.htm

[3]http://styx.nsci.plu.edu/~dhansen/circulation.pdf

[5]Mintun M.A., Lundstrom, B.N., Synder, A.Z., Vlassenko A.G., Shulman G.L., and Marcus Raichle. "Blood flow and oxygen delivery to human brain during functional activity: Theoretical modeling and experimental data." PNAS 2001. 98 (12) 6859-6864.

[5]Tsai A.G., Friesenecker B., Mazzoni M.C., Kerger H., Buerk D.G., Johnson P.C., and M. Intaglietta. "Microvascular and tissue oxygen gradients in the rat mesentery." PNAS 1998. 95 (12), 6590-6595

[4]Weind, KL, Boughner DR, Rigutto L, and CG Ellis. "Oxygen diffusion and consumption of aortic valve cusps" Am J Physiol Heart Circ Physiol 281: H2604-H2611, 2001.

Postmortem:

There were considerable difficulties in obtaining the solution. However, the problem stemmed mostly from the initial simplification process of the model, and not on the program FIDAP itself. Most importantly, the conflicting results between our expected and actual results stumped the progress. Initially, we predicted that convection by the fluid would be the major factor in transporting oxygen in the capillary, while diffusion is the dominant force once the oxygen reaches the liver tissue. However, we obtained a very unique solution whereby the oxygen source at the inlet seemed to behave as a point source.

After considerable work, we discovered that diffusion is the major force for the oxygen in the fluid, not convection of the moving fluid down the capillary. Because the convection is minimal compared to the diffusion, all of the oxygen is diffused before the fluid has a chance to pull the oxygen down the capillary. This result was a huge surprise that took us days to unravel.

In light of understanding the result, the root of the problem was in the simplification of the problem. In nature, the oxygen in the blood is not carried as diffusing species in the capillary. Instead, the oxygen is tightly bound to hemoglobin *inside* the red blood cells, and thus, this system prevents the straight diffusion as taking the dominant role of oxygen transport. This hemoglobin release of oxygen is dependent on many factors that were not included in the simulation, such as the pH gradient, CO_2 gradient and other physiological factors. Hence, oxygen transport to targets far away can be accomplished, compared to 60 μm.

In addition, sensitivity analysis shows that the results obtained are very sensitive to the constants and constraints placed upon our model. Hence, accurate values must be experimentally determined. Currently, there is a lack of research depth in tissue engineering, and thus, accurate values of liver density and liver tissue metabolic rate, for example, could not be obtained

Overall, this project has showed us that what we see can be deceiving. We should always be open to results that skew from the prediction and most importantly, if you check everything many times and there is nothing wrong with the input, there must be no problem with the output.